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INFRARED SPECTRA OF PLASMA MEMBRANE AND ENDOPLASMIC RETICULUM OF EHRLICH ASCITES CARCINOMA

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SUMMARY

The infrared spectra of dried films of plasma membrane and endoplasmic reticulum are dominated by the Amide I and II bands of membrane proteins and by the O–H, C–H, C=O, P=O, C–O–C and P–O–C vibrations of membrane lipids. The two membrane types differ in their lipid–protein associations. High amidation of the proteins of both membranes is indicated by the pH-dependent spectral changes and also by amino acid analysis. Exposure of the membranes to acid pH produces spectral evidence for rearrangement of some membrane peptide into β-conformation.

INTRODUCTION

The infrared spectra of several cellular membranes have been published recently[1–5]. Detailed analyses have been limited to the Amide I and Amide II regions of spectra obtained on erythrocyte ghosts[2,3] and plasma membranes of Ehrlich ascites carcinoma[5], and suggest that the proteins of these membranes lack β-conformations. We now compare the infrared spectra of plasma membrane of Ehrlich ascites carcinoma with those of endoplasmic reticulum and correlate the bands between 4000 and 700 cm^{-1} with the composition and ionic characteristics of these membranes.

EXPERIMENTAL

Materials

Plasma membrane and endoplasmic reticulum vesicles, about 1500 Å in diameter[6] were prepared from Ehrlich ascites carcinoma microsomes as previously described[7,8]. To eliminate the sucrose or polysucrose used in their preparation, the membranes were washed once with 0.01 M $CaCl_2$, 0.02 M Tris–HCl (pH 8.2), and once with 0.01 M Tris–HCl (pH 8.2). Centrifugations were at 50000 rev./min for 30 min at 4° in a Spinco SW50 rotor. The pellets of the second wash were then dispersed uniformly in water to a concentration of about 1 mg protein/ml. Protein determinations on the final membrane dispersions or solutions were by both the ninhydrin procedure[9] and the method of LOWRY *et al.*[10], using crystalline bovine serum albumin as reference standard. The ninhydrin procedure gave 'protein' values which were 10 % higher than that obtained by the LOWRY method. Total lipid was determined on

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extracts prepared by the method of FOLCH, LEES AND SLOANE-STANLEY[11]. Measurements of cholesterol and lipid phosphorus were according to the methods of SEARCY AND BERGQUIST[12] and LOWRY *et al.*[13], respectively.

Amino acid analyses were on membranes, extracted according to FOLCH, LEES AND SLOANE-STANLEY[11], hydrolyzed with 6 M HCl in sealed evacuated tubes at 105° for 16 h. A Beckman Spinco amino acid analyzer was employed. Tryptophan was estimated spectrophotometrically[14] on ribonuclease-treated membranes dissolved in 1 % sodium lauryl sulfate.

One mg of membrane 'protein' corresponds to about 1.5 mg dry weight, most of the non-protein mass consisting of phosphatides and cholesterol. One to 2 % of the dry mass of plasma membrane and 3–4 % of the endoplasmic reticulum is due to tightly bound RNA. These estimates are based on the ribose content[15] and ultraviolet spectra of washed, phenol-extracted membranes. It should be noted that cellular membranes obtained in isoosmotic media (*e.g.*, 0.25 M sucrose) ordinarily contain considerable quantities of trapped, soluble proteins, but in the isolation procedure here employed, these contaminants were removed by 'osmotic shock'[7,8,16]. The reported spectra are thus due to components integral with the membrane structure.

Infrared spectra

A Perkin–Elmer spectrophotometer Model 521 was employed. Solid films were prepared by applying about 0.5 mg membrane protein, in aqueous suspension, as a 0.5 cm × 2 cm band in the center of the AgCl plate and drying in air at 25°. Once dried, the films were strongly adherent to the plate. Extraction of lipids from the films was achieved by immersing the plates in acetone–water (9:1, v/v) or chloroform–methanol (2:1, v/v) for 20 min at room temperature. After rinsing with the same solvent, the films were dried in air. To acidify the films, the dried plates were immersed for 20 min in 0.001–0.1 M HCl at room temperature, rinsed with distilled water, and air-dried. To return to alkaline pH, the films were immersed in 0.05 M Tris–HCl (pH 8.2) for 20 min, rinsed with distilled water, and air-dried.

For neuraminidase treatment the films were immersed in 0.01 M acetate buffer, 0.01 M in $CaCl_2$ and 0.05 M in NaCl, containing 25 units/ml of crystalline neuraminidase from *Vibrio cholerae* (Behring Werke, Marburg-Lahn, Germany) for 1 h at 37° in a moist chamber. After treatment the films were rinsed with 0.05 M NaCl, then distilled water, and air-dried. 0.028 μmole neuraminic acid is released per mg plasma membrane protein and 0.016 μmole per mg endoplasmic reticulum protein.

In order to evaluate changes in the film absorbances in a meaningful way, it was necessary to have the films located reproducibly in the optical path. For this reason all manipulations of the films were performed with the plates in their plateholders. With this technique protein losses from the films were negligible, judging by the absorbances of the Amide I and II bands.

RESULTS

Origins of the infrared spectra of endoplasmic reticulum and plasma membrane

The 2000–700 cm^{-1} region of an endoplasmic reticulum film spectrum, before and after extraction of lipid, is reproduced in Fig. 1. The various absorption bands, their assignments[17] and their tentative origins are listed in Table I.

TABLE I

PRINCIPAL BANDS SEEN IN THE INFRARED SPECTRA OF PLASMA MEMBRANE AND ENDOPLASMIC RETICULUM

Frequency (cm^{-1})	*Principal vibration*	*Effect of lipid extraction*	*Tentative origin*
3400	O–H stretch	Slightly reduced	Cholesterol: hydroxy amino acids; RNA; carbohydrate; H_2O
3000–2800	C–H stretch	Much reduced	All membrane components
3060	$-NH_3^+$ stretch	No effect	Phosphatidyl ethanolamine; phosphatidyl serine; side-chain and terminal amino groups of protein
1750–1710	C=O stretch	Much reduced	Ester groups of phosphatides; unionized carboxyl groups
1650	Amide I (C=O stretch)	Slightly reduced	Peptide (α-helix or 'unordered'); amide; sphingomyelin
1590	$-CO_2^-$; asymmetric stretch	Slightly reduced	Ionized carboxyl groups
1540	Amide II (N–H bending)	Slightly reduced	Peptide; sphingomyelin
1465–1445	Methylene scissoring and asymmetric methyl bending	Much reduced	All membrane components
1400	$-CO_2^-$ stretch; symmetric	Slightly reduced	Ionized carboxyl
1230	P=O stretch C–O–C stretch (asymmetric)	Much reduced	Phosphatides; RNA
1170	C–O–C stretch (symmetric)	Abolished	Phosphatide
1080–1060	P–O–C stretch	Much reduced	Phosphatide; RNA
970	C–H; out of plane deformation	Abolished	*Trans*-unsaturated fatty acids; also in all lecithins
720	C–H; rocking	Abolished	Hydrocarbon chain of lipids

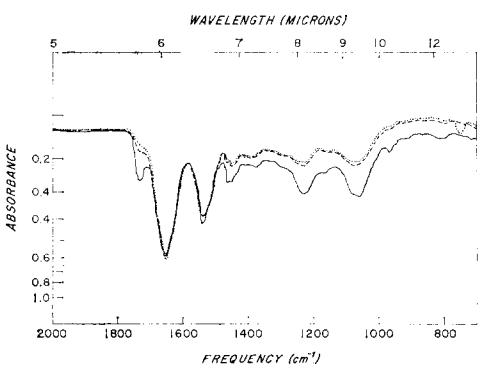


Fig. 1. Infrared spectrum of an endoplasmic reticulum film. ———, untreated; — — —, after extraction with acetone–water (9:1, v/v); ·····, after extraction with chloroform–methanol (2:1, v/v).

Differences between the spectra of endoplasmic reticulum and plasma membrane

The infrared spectra of plasma membrane prior to lipid extraction are not noticeably distinct from those of endoplasmic reticulum. However, treatment with lipid solvents uncovers some noteworthy differences between the two membrane types:

Lipid extractability

Extraction of endoplasmic reticulum and plasma membrane films with chloroform–methanol (2:1, v/v) completely eliminates the lipid contribution to the spectrum (Fig. 1), as borne out by direct chemical analysis. Extraction with acetone–water (9:1, v/v) is almost equally effective in the case of endoplasmic reticulum (Fig. 1),

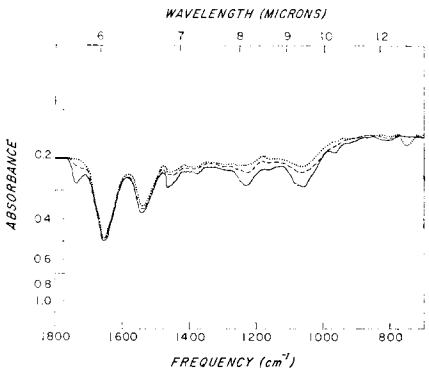


Fig. 2. Infrared spectrum of a plasma membrane film. ———, untreated; — — —, after extraction with acetone–water (9:1, v/v); ·····, after extraction with chloroform–methanol (2:1, v/v).

but not in the case of plasma membrane (Fig. 2). Chemical analyses show that this solvent mixture extracts over 90 % of the lipid phosphorus from endoplasmic reticulum and only 50–60 % from plasma membrane. However, acetone–water (9:1, v/v) extracts most of the cholesterol from both types of membrane. The small band at 750 cm^{-1} appearing after extraction with chloroform–methanol is attributed to C–Cl stretching of residual chloroform. It disappears after washing with acetone or aqueous buffers.

pH-dependent spectral changes

The infrared spectra of plasma membrane and endoplasmic reticulum, extracted at neutral pH, differ noticeably in the C=O stretching region (1750–1710 cm^{-1}). In plasma membrane this region is featureless after lipid extraction (Fig. 2), whereas the endoplasmic reticulum spectra have a pronounced shoulder there (Fig. 1). The origin of the residual band in endoplasmic reticulum is uncertain.

Exposure of lipid-extracted films to 0.1 M HCl increases the absorbance at 1720 cm^{-1} in both membrane types, *i.e.* greater prominence of the shoulder in the endoplasmic reticulum spectra and appearance of a shoulder in the plasma membrane spectra. This is accompanied by a decrease of absorbance near 1590 cm^{-1}, the region of asymmetric stretching of CO_2^- groups. If the films are now washed with 0.05 M Tris–HCl (pH 8.2), the shoulder disappears from the plasma membrane spectra and returns to its original value in the endoplasmic reticulum spectra. The pH-dependent absorbance changes at 1720 and 1590 cm^{-1} are thus attributable to carboxyl groups, including neuraminic acid residues, side-chain amino acid carboxyls and terminal amino acid carboxyls.

TABLE II

AMINO ACID COMPOSITION OF SOME MEMBRANE PROTEINS

Values presented are the number of residues per 100 residues.

Amino acid	*Endoplasmic reticulum**	*Plasma membrane**	*Liver bile fronts***	*Liver Smoot microsomal membranes****	*Mitochondrial structural protein*§	*Myelin*§§
Lys	6.5	6.3	7.2	6.3	6.3	5.8
His	2.1	2.6	2.6	—	1.2	2.3
Arg	5.2	4.7	5.2	3.6	4.1	4.0
NH_3	10.8	14.7	12.4	15.3	10.1	·
Asp	8.7	8.8	9.3	10.0	6.6	6.1
Glu	10.6	10.1	12.0	10.6	6.2	7.1
Thr	5.4	5.5	5.3	5.4	4.2	6.4
Ser	6.2	6.6	6.0	4.4	4.2	10.4
Pro	5.4	5.2	4.9	6.6	2.3	1.1
Cys	trace	trace	0.9	0.06	7.7	3.8
Met	2.5	2.7	2.3	—	2.0	1.0
Gly	7.7	8.5	7.8	7.3	7.8	10.9
Ala	7.6	7.8	8.0	7.2	8.4	9.6
Val	6.7	6.6	6.6	6.5	5.4	5.9
Ile	5.1	6.1	5.1	4.7	5.0	4.5
Leu	10.0	10.1	9.6	9.9	7.8	8.8
Tyr	3.4	3.1	2.7	—	3.2	3.1
Phe	4.8	4.8	4.5	2.8	4.4	4.0
Trp	1.5	1.5	· ·	0.2	3.2	5.1

* Values of NH_3, Ser, Thr, Tyr and Trp corrected for decomposition of these amino acids during hydrolysis.
** Calculated from ref. 21.
*** Calculated from ref. 22.
§ Calculated from ref. 24.
§§ Calculated from ref. 1.

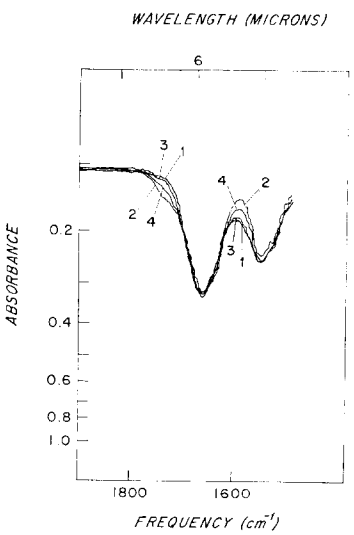


Fig. 3. Effects of acid pH and neuraminidase treatment upon the infrared spectrum of a lipid-free plasma membrane film. 1. Film after extraction with chloroform–methanol (2:1, v/v). 2. Same after washing with 0.01 M HCl. 3. After neuraminidase treatment. 4. Neuraminidase-treated film washed with 0.01 M HCl. Note the different amplitudes of the pH-dependent changes at 1720 and 1590 cm^{-1} before (Curves 1 and 2) and after (Curves 3 and 4) neuraminidase treatment.

Spectra were also obtained after exposure of films, extracted at neutral pH, to solutions of varying acidity. These show that the absorbance changes at 1720 and 1590 cm^{-1} occur between pH's 4 and 2, consistent with their assignment to carboxyl groups.

Exposure of both membranes to acid pH has, as a second consequence, the appearance of a shoulder near 1630 cm^{-1}. This change, which is due to a refolding of some of the membrane peptide into a β-conformation[5] is not reversed by washing the films with buffers of neutral or alkaline pH.

The possible origins of the reversible, pH-dependent absorbance changes are: (a) neuraminic acid (pK 2.6), of which there is about 0.0028 μmole/mg of plasma membrane protein and 0.016 μmole/mg of endoplasmic reticulum protein[18]; and (b) γ-carboxyls of aspartic and glutamic acids, the sum of which is about 1.4 μmoles/mg protein in both membrane types (Table II).

In the case of plasma membrane some of the pH-dependent changes are attributable to the titration of neuraminic acid, as shown by their diminution after treatment of plasma membrane films with neuraminidase (Fig. 3). As expected from its lesser neuraminate content, neuraminidase treatment of endoplasmic reticulum produces only barely detectable changes in the 1720 and 1590 cm^{-1} regions.

The C=O stretching bands are rather strong, as can be seen from the absorbance decrement at 1750–1710 cm^{-1} after extraction of phosphatides (about 0.5 μmole glycerophosphatide or 1 μmole C=O per mg protein). Moreover, titration of 0.03 μmole of neuraminic acid per mg protein is clearly detectable. In contrast, the pH changes which should protonate 1.4 μmoles of side-chain carboxyl per mg protein cause little change of absorbance at 1720 and 1590 cm^{-1}. Although one cannot expect good adherence to the Beer–Lambert law in film spectra, it is most likely that the observed discrepancy reflects the high degree of amidation of membrane proteins also implicit in the large ammonia values found upon amino acid analysis. Accordingly, a certain portion of the absorbance in the Amide I region is attributable to glutamine and asparagine.

Amino acid analyses

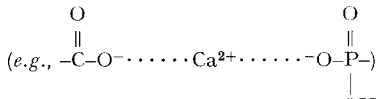
The amino acid analyses of lipid-free endoplasmic reticulum and plasma membrane are summarized in Table II. The values presented are averages of duplicate determinations on two preparations of each membrane type, and are corrected for the decomposition of serine, threonine, tryptophan and tyrosine during hydrolysis[19]. The maximum deviation between two membrane preparations was in the ammonia values of plasma membrane, which varied over a range of $\pm$ 12 % despite meticulous care to avoid contamination by extraneous ammonia.

DISCUSSION

The infrared spectra of dried plasma membrane and endoplasmic reticulum films are much alike, the prominent features of both being the Amide I and Amide II bands of membrane proteins and the C–H, O–H, C=O, P=O, C–O–C and P–O–C vibrations of the membrane lipids. However, extraction of the films with acetone–water (9:1, v/v) shows that the lipid–protein associations of endoplasmic reticulum and plasma membrane differ considerably. In this respect the endoplasmic reticulum

membranes are more akin to mitochondria, whose phosphatides are also almost fully extracted by acetone–water (9:1, v/v)[20].

The pH-dependent changes in the infrared spectra of lipid-free membranes suggest that their proteins are highly amidated and this is also indicated by the high yields of ammonia in the amino acid analyses. High ammonia values are also reported for the bile front membranes[21] and smooth microsomal membranes[22] of rat liver. According to the amino acid analyses, there is about 1 μmole of basic amino acid per mg protein in endoplasmic reticulum or plasma membrane but only about 0.6 μmole of unamidated side chain carboxyl in endoplasmic reticulum and 0.3 μmole in plasma membrane, suggesting that the proteins of both membranes carry a net positive charge at neutral pH. However, since we have shown[6] that the intact membranes carry an excess negative charge above pH 4.5 (even after removal of neuraminic acid[18]), we suspect ion-pairing between basic amino acids and lipid phosphate. Extensive ion triplet formation



which has been invoked in the association of membrane lipids with proteins[23], is not consistent with the data. Whatever the ionic interactions between membrane proteins and lipids, they must be relatively weak, since the lipids are readily extracted by certain solvents whose low dielectric constants favor ionic interactions.

Since endoplasmic reticulum and plasma membrane obviously have multiple protein constituents, their amino acid analyses have limited value and were undertaken only to test for possible gross compositional features distinctive of endoplasmic reticulum, plasma membrane or membrane proteins in general. The amino acid compositions of endoplasmic reticulum and plasma membrane do not differ significantly and are almost identical to that of liver bile front membranes[21]. Moreover, these three membrane types and the smooth microsomal membranes of rat liver[22] have a very different amino acid makeup from that of mitochondrial structural protein[24] and myelin[1] (Table II). Particularly noteworthy are their higher proportions of proline and aspartic acid + glutamic acid and their very low (half)cystine values. The latter indicate that disulfide bridges are not a significant feature in the protein architecture of plasma membrane, endoplasmic reticulum, bile front membranes and the smooth microsomal membranes of liver. The proline content of plasma membrane and endoplasmic reticulum is very similar to that of hemoglobin (5.5 moles %), a highly helical protein, and is thus not incompatible with the proposal, that there is considerable α-helix in many membranes[5,25]. It is clear in any event that mitochondrial structural protein and the proteins of myelin are not representative of membrane proteins in general.

It is of considerable interest that protonation of the protein carboxyls of endoplasmic reticulum is associated with some transition to β-conformation. This is also found in plasma membrane and in both membranes is more prominent after exposure to acidic organic solvents (acid 2-chloroethanol, formic acid, acetic acid), in which the membrane proteins have appreciable solubility. This phenomenon is under further study.

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